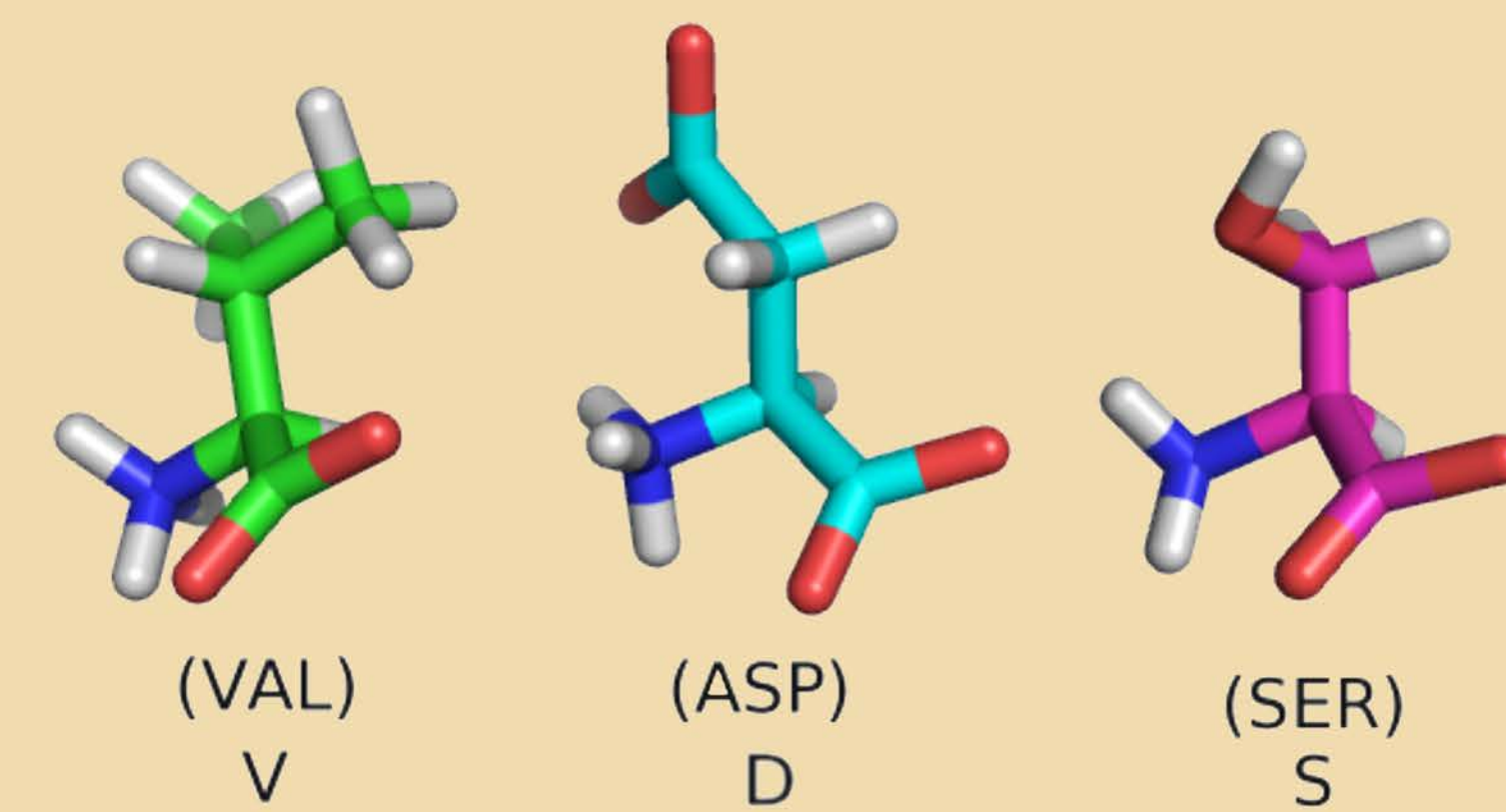


Discovery of Novel Ligands Inhibiting the DHFR Active Site of Bifunctional Dihydrofolate Reductase-Thymidylate Synthase (DHFR-TS) of *Leishmania major* Using GOLD Virtual Screening

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Abstract

The *Leishmania major* parasite is the causative agent of leishmaniasis, a neglected tropical disease that affects over one million people worldwide. This study focused on the use of virtual screening to find novel inhibitors of the *L. major* DHFR-TS enzyme essential to the organism's survival. The desired gene was cloned through PCR-based gene synthesis and ligation of the product into a pET vector. An *E. coli* DHFR surrogate enzyme was expressed with IPTG, and purified using nickel affinity chromatography and size-exclusion FPLC. Enzyme activity of surrogate enzyme phosphatase YopH was validated by performing enzyme activity assays. The inhibitory action of compounds was tested against YopH using enzyme inhibition assays. Various compound libraries were virtually docked in the active site of DHFR-TS using the GOLD program, returning fitness scores indicating possible inhibitory action. Compounds exhibiting encouraging *in silico* results for DHFR-TS were selected as being potential *in vitro* inhibitors of the enzyme, but could not be validated as such because of unsuccessful enzyme expression. The primary goal of this research was unsuccessful, and further experimentation on the actual drug target must be conducted.

Introduction

Disease of Interest: Leishmaniasis

- Leishmaniasis is neglected tropical disease that causes tissue necrosis¹
- Caused by *Leishmania major* – a vector-borne, unicellular, eukaryotic parasite
- Transmitted from host to host through the bite of the sand fly
- Individuals afflicted with the disease experience a decreased quality of life and will often die from a lack of effective treatment
- In developing countries, leishmaniasis and other neglected tropical diseases are prevalent causes of high mortality rates and economic adversity²
- Current treatments rely on toxic antimonials with an unknown mode of action³

Target Enzyme: DHFR-TS

- Protists synthesize DHFR-TS, a dimer of dihydrofolate reductase and thymidylate synthase⁴
- Catalyzes various steps of the thymidylate cycle, the cell's only *de novo* source of dTMP (Fig. 6)
- Reduces dihydrofolate to tetrahydrofolate with NADPH as a cofactor (Fig. 3)
- Inhibiting DHFR activity leads to cytotoxicity because DNA biosynthesis cannot take place⁵
- DHFR of *H. sapien* and *L. major* have a 26% identity match, suggesting any ligands binding to *L. major* DHFR-TS would not cause side effects in *H. Sapien* DHFR⁵

Materials and Methods

Virtual

- Attained virtual model of *L. major* DHFR-TS from David A. Matthews⁵
 - Analyzed the structure's binding sites using PyMOL visualizer (Schrödinger, USA)
- Used GOLD⁶ program (CCDC, Cambridge, England) to dock compounds into enzyme's active site
 - Conducted several trial dockings to optimize active site coordinates and validate GOLD as an accurate method of virtual screening against DHFR-TS
 - Conducted docking of novel ligands using various compound libraries
 - Filtered the highest scoring compounds using Lipinski's Rule⁷ and PyMOL to identify those with drug-like qualities

Gene Cloning

- Codon optimization⁸ of *L. major* DHFR-TS gene for expression in *E. coli*
- Oligonucleotide primer sequences and custom forward and reverse primers ordered from Integrated DNA Technologies, Inc. (IDT, Coralville, IA)
 - 38 oligonucleotide sequences with complementary overhangs
- PCR-based gene synthesis created the desired *L. major* DHFR-TS gene
 - Primary PCR – annealed oligonucleotides to each other
 - Secondary PCR – selectively amplified full-length gene from primary PCR product using custom forward and reverse primers
 - PCR² – further amplified full-length gene from secondary PCR product
- Ligation independent cloning for the insertion of the synthesized gene into the pNIC28-Bsa4 vector
 - Restriction enzyme digest created complimentary overhangs on the vector and gene
 - Annealed vector to gene at overhangs
- Vector transformed into competent *E. coli* DH5a cells
 - Ideal for DNA replication
- Plasmids isolated from various DH5a colonies
 - Sequenced at the ICMB Core Sequencing Facility to determine if a positive cloned was attained

Protein Expression, Purification, and Characterization

- Vector transformed into *E. coli* BL21(DE3) cells
 - Optimal for protein expression
- Isopropyl β-D-1-thiogalactopyranoside (IPTG) used for the induction of protein expression
- Cells lysed by sonication
- Performed Ni-NTA column chromatography purification
 - Exploitation of the histidine tag on the protein encoded by the pNIC-Bsa4 expression vector
- SDS-PAGE gel (Bio-Rad, Hercules, CA) used in the characterization of the protein

Enzyme and Inhibition Assays

- Mechanism of action validated through an enzyme activity assay
- Inhibitory activity validated through an enzyme inhibition assay
 - Run with known inhibitors and highest scoring compounds from virtual screening

Figure 1. Model of DHFR-TS complexed with NAPDH, CB3, CYF, and Repaglinide; DHFR-TS as grey surface, compounds as black sticks, carbon as yellow, oxygen as red, nitrogen as blue

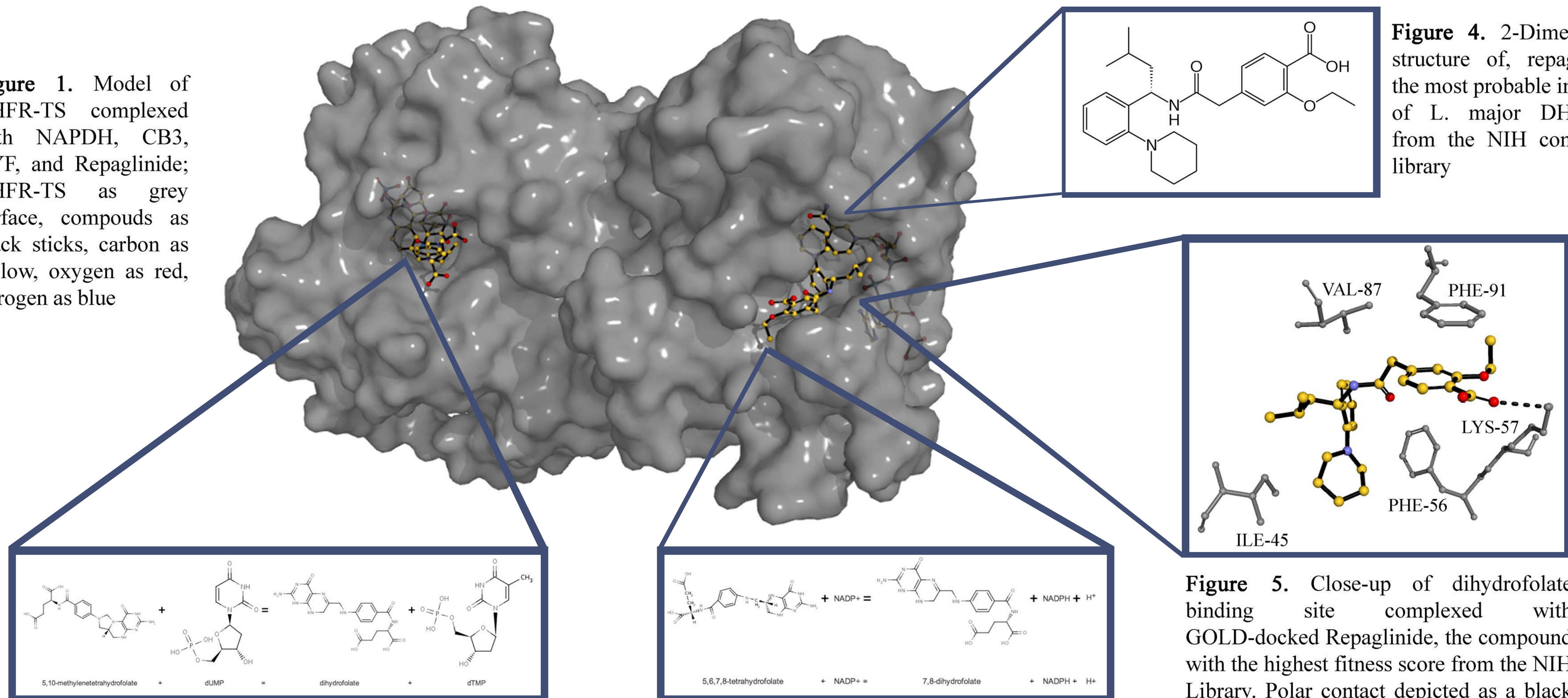


Figure 2. Reaction catalyzed by thymidylate synthase (2.1.1.45)

Figure 3. Reaction catalyzed by dihydrofolate reductase (1.5.1.3)

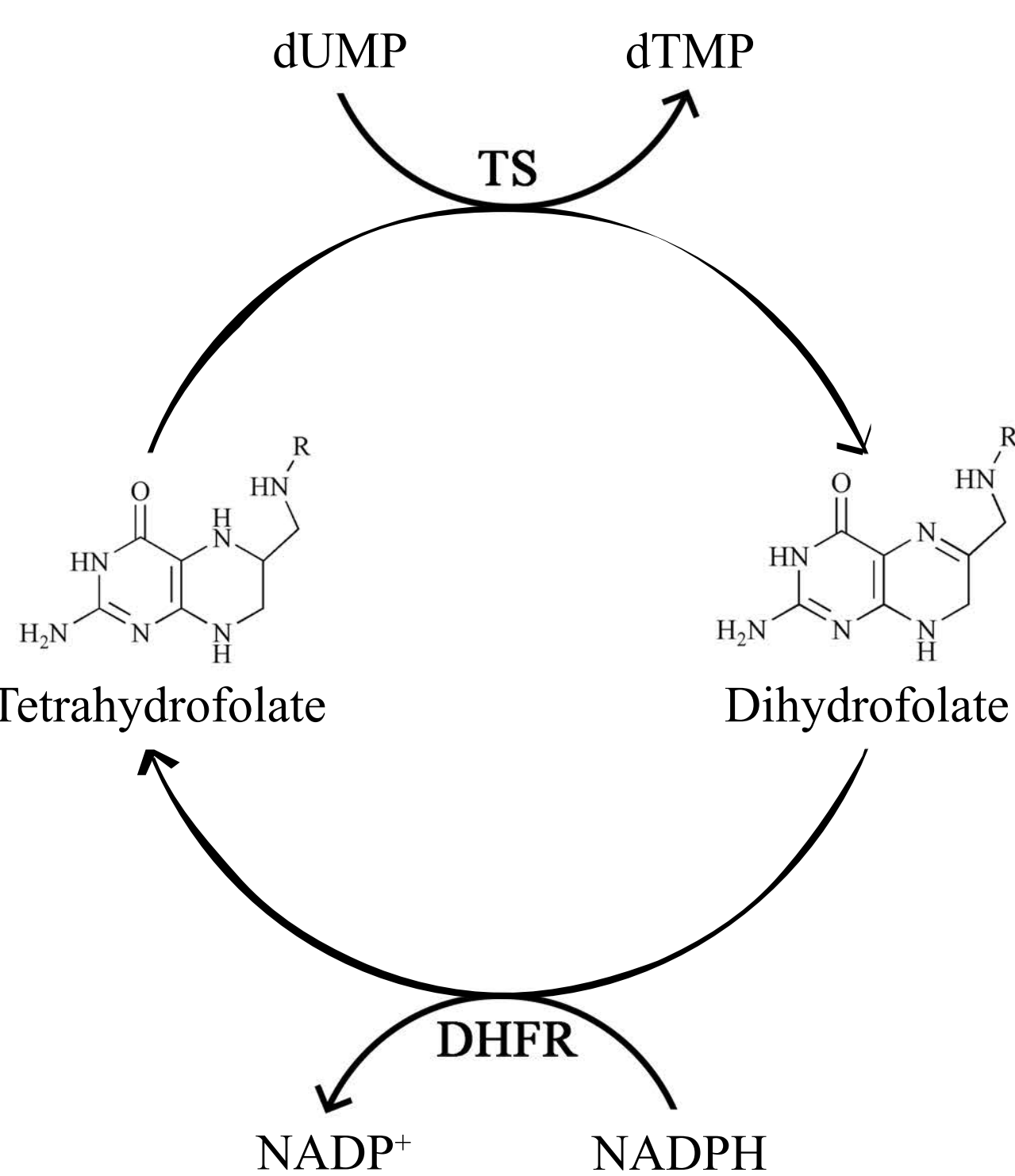


Figure 6. Reaction diagram of the thymidylate cycle; TS: thymidylate synthase, DHFR: dihydrofolate reductase

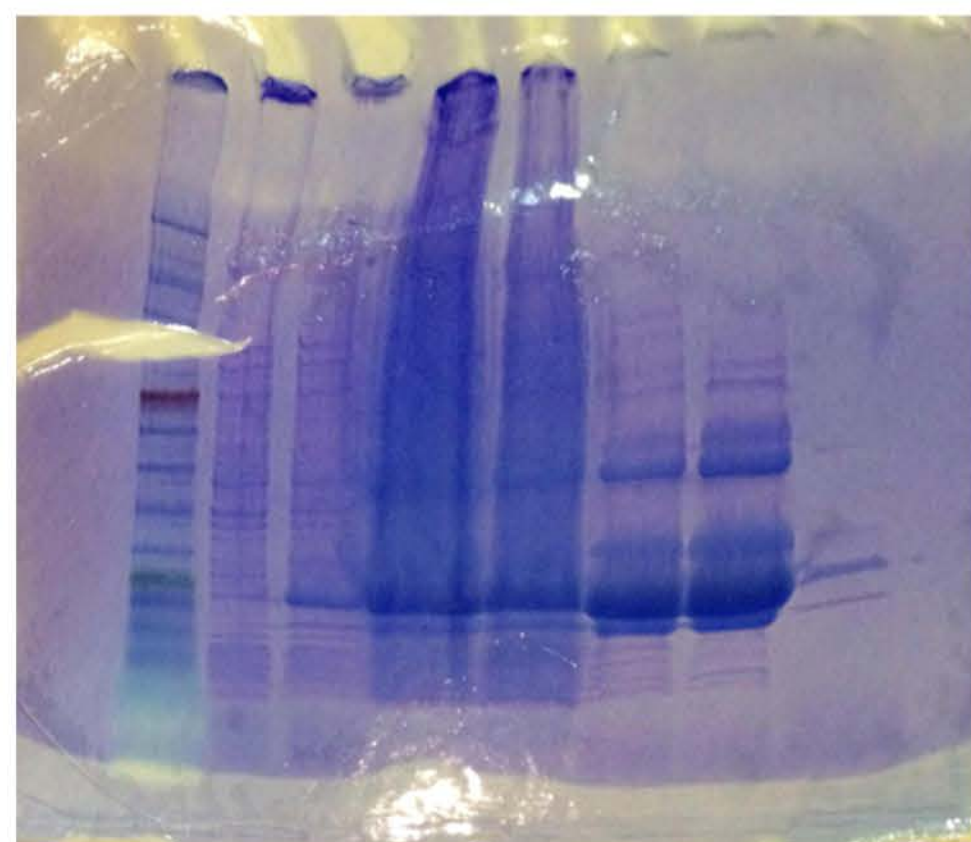


Figure 9. SDS-PAGE Gel Electrophoresis of His-Tag purified *E. coli* DHFR; Well 1: Thermo Scientific Prestained Protein Ladder 10-170kDa; Well 2: Cell lysate before induction; Well 3: Cell lysate after induction; Well 4: Supernatant of Lysed Cells; Well 5: Flowthrough of supernatant flushed with Ni-NTA resin/buffer mix; Well 6: Wash fraction with 20 mM Imidazole; Well 7: Elution 1, using 250mM Imidazole; Well 8: Elution 2, using 250mM Imidazole

Score	Ligand Name	S (PLP)	S (Hbond)	S (cho)	DE (clash)	DE (tors)	Intoor
94.22	SAM001246195	-94.65	0	0	0	0	6.07
91.19	SAM001246588	-92	0.46	0	0	4.02	3.87
90.98	SAM001246637	-88.02	1.59	0	0	2.95	4.09
90.61	SAM001246546	-85.9	2	0	0	2.17	3.54
89.35	SAM001246657	-91.06	0	0	0	2.93	3.18
88.5	SAM001246577	-89.34	0.13	0	0	2.44	3.28
87.44	SAM001246736	-84.34	1.36	0	0	0.9	1.22
87.64	SAM001246532	-84.23	1.7	0	0.65	0.94	0.84
87.49	SAM001246985	-76.23	5.25	0	0	2.66	0.83
87.29	SAM001246796	-71.38	5.93	0	0	1.39	0.6
86.51	SAM001246598	-83.37	1.05	0	0	1.67	3.59
85.92	SAM001246602	-82.82	1.52	0	0	2.77	4.08
84.97	SAM001246968	-82.42	1.75	0	1.99	1.93	2.79
83.94	SAM001246860	-78.63	1.94	0	0	1.38	1.44
83.13	SAM001246581	-73.67	3.52	0	0	2.76	4.43
83.12	SAM001246727	-80.17	1	0	0	0.75	1.45
82.71	SAM001246712	-80.32	0.93	0	0	0.84	3.2
81.99	SAM001246965	-92.67	0	0	0	0.76	0.85

Figure 7. GOLD docking of the NIH compound library into the DHFR active site of *L. major* DHFR-TS; ranked from highest to lowest GOLD fitness score; compounds adhering to Lipinski's Rule shown in green, compounds not adhering to Lipinski's Rule shown in red

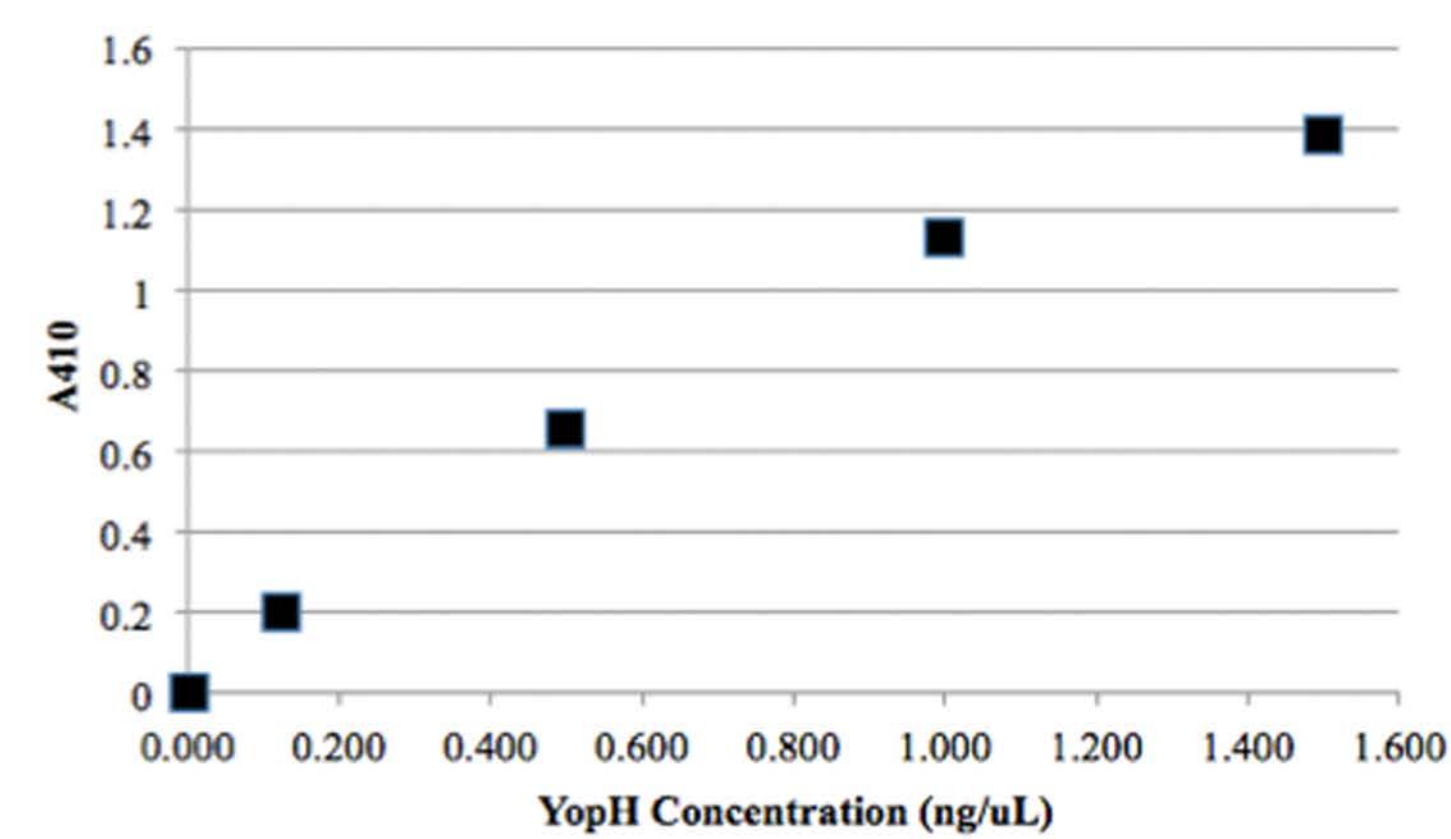


Figure 10. Enzyme assay testing *Y. pestis* YopH activity. YopH Concentration (ng/uL) vs absorbance of pNP at 410nm (n=1)

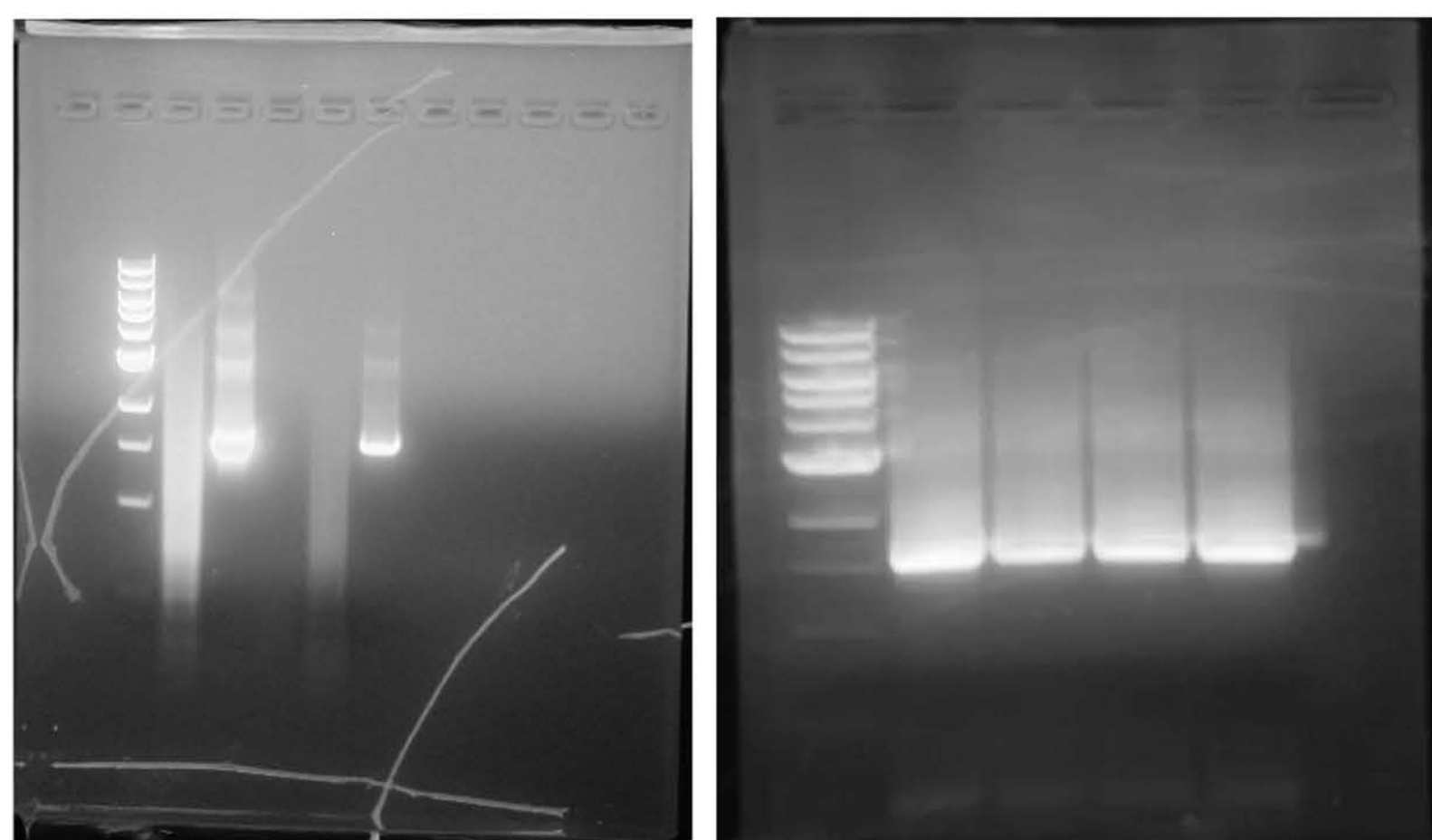


Figure 8. Left: Overlap PCR of *L. major* DHFR-TS; Lane1: 100bp Ladder, Lane 2: 1° PCR, Lane 3: 2° PCR, using custom forward and reverse primers, Lane 5: (2) 1° PCR, Lane 6: (2) 2° PCR, using custom forward and reverse primer. Right: PCR² of Secondary PCR Product; Lane 1: NEB 1kb ladder; Lane 2, 3, 4, 5: PCR² product; Lane 6: Empty

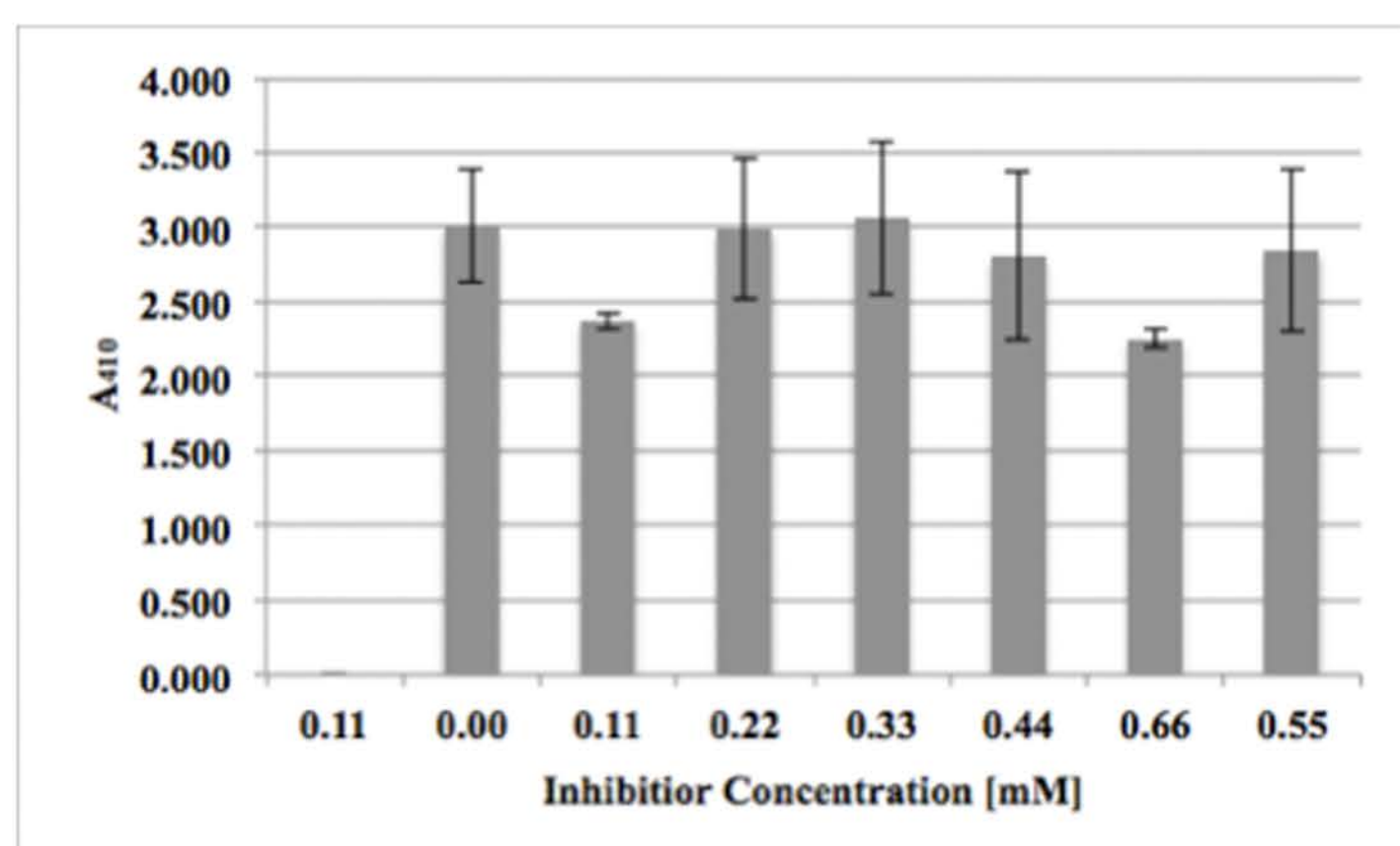


Figure 11. Enzyme inhibition graph of *Y. pestis* YopH using compound #5250098 at various concentrations; leftmost column: control using no enzyme; rightmost column: positive control using known inhibitor sodium orthovanate (n=2)

Results

Virtual Screening

- GOLD was validated as an accurate docking program for the *L. major* DHFR-TS enzyme to ensure that the reported fitness scores were dependable
 - Ligands known to bind with high and poor affinity within the DHFR active site were selected as controls in the run
- Because cloning was unsuccessful, virtual screening was limited to only screening one library against the provided DHFR-TS structure
 - The NIH Library, composed of 446 ligands, was docked into the DHFR active site (Fig. 7)
 - The highest-scoring ligand with an acceptable adherence to Lipinski's Rule was SAM001246546, or repaglinide, with a fitness score of 90.61 (Fig. 4)
 - PyMOL visualization of the repaglinide docking in the DHFR active site indicated hydrophilic interactions of the ligand with residue LYS-57, and hydrophobic interactions with residues ILE-45, PHE-56, VAL-87, and PHE-91 (Fig. 5)
- The high scores of top novel ligands exhibit the potential to be *in vitro* inhibitors

Gene Cloning

- Primary PCR showed a smear, indicating the synthesis of variable-length molecules (Fig. 8)
- Secondary PCR showed a bright band at ~1500bp, indicating a successful amplification of the gene of interest by the custom tail primers (Fig. 8)
- PCR² amplification was validated on a 1% agarose gel stained with ethidium bromide (Fig. 8)
- PCR² samples were ligated into plasmids and sequenced to validate identity
- A positive clone with 100% query coverage and identity was never attained

Protein Expression, Purification, and Characterization

- Cloning was unsuccessful and attention was redirected towards *E. coli* DHFR as a surrogate enzyme
- E. coli* DHFR was expressed, purified, and visualized on an SDS-PAGE gel; a band at 18kDa was seen (Fig. 9)
- Enzyme was further purified through fast protein liquid chromatography

Enzyme and Inhibition Assays

- Attention was redirected towards working with a phosphatase YopH from *Yersinia pestis*
- An analysis of the enzyme assay data indicated that 1 ng/μL was the ideal concentration of enzyme to be used in inhibition assays (Fig. 10)
- Compound #5250098 was tested for inhibitory action against the previously validated YopH surrogate enzyme (Fig. 11)

Conclusions and Future Directions

- Because a positive clone was not attained, and *L. major* DHFR-TS expression was not possible no conclusions can be drawn about *in vitro* inhibition of the enzyme. Virtual screening of the NIH library against DHFR-TS showed promising results of repaglinide as a possible inhibitor of the enzyme.
- The immediate next step is to order IDT gBlocks[®] of ~900bp in length that can be joined via Gibson Assembly[®] Method⁹ to give a complete DHFR-TS sequence that can be ligated into an accepting vector. The enzyme could also be purified using a methotrexate-Sepharose affinity column and then eluted with dihydrofolate⁶.
- Different compound libraries will be docked against the current crystal structure in order to determine which compounds could serve as starting points for strong inhibition of the *L. major* DHFR-TS enzyme.
- More attention will be placed on inhibition of the thymidylate synthase active site (Fig. 2). If both regions of the dimer could be inhibited simultaneously, a more complete inactivation of the thymidylate cycle could be achieved.
- The ultimate goal of research on *L. major* DHFR-TS is to crystallize and publish the structure in the Protein Data Bank. Ideally, *L. major* DHFR-TS would be complexed with any promising inhibitors. Any resulting structures could be used for further virtual screening.

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